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W81XWH-14-1-0483

TITLE:
"Recovery of Breathing and Forelimb Function after Prolonged Exposure to Repetitive Acute Intermittent Hypoxia".

PRINCIPAL INVESTIGATOR: Gordon S. Mitchell, PhD

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14. ABSTRACT
The fundamental goal of this project is to test the efficacy and safety of prolonged repetitive exposure to acute intermittent hypoxia (rAIH; 10 episodes per day, 3 to 4 days per week, 3 to 6 months) in a rodent model of chronic, incomplete cervical spinal injury (C_2 spinal hemisection in rats; C2HS). In this collaborative project (Florida and Saskatoon, Canada), we are exploring the impact of prolonged rAIH on both respiratory (Florida) and limb function (Canada), and on markers of neuro-cognitive and cardiovascular safety (Florida). Three specific aims were proposed: **Aim 1**: Test the hypothesis that prolonged rAIH elicits robust and prolonged improvement of breathing capacity after chronic C2HS; **Aim 2**: Test the hypothesis that prolonged rAIH in combination with task specific training elicits robust and prolonged improvement of voluntary forelimb function after chronic C2HS; and **Aim 3**: Test the hypothesis that prolonged rAIH has no significant impact on hippocampal cell survival or systemic blood pressure. These pre-clinical studies are an essential "next-step" in our efforts to translate rAIH as a therapeutic modality to restore respiratory and non-respiratory motor function in patients with chronic, incomplete SCI.

15. SUBJECT TERMS
Spinal cord injury, treatment, intermittent hypoxia, prolonged, rats, safety, efficacy.

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TABLE OF CONTENTS

PROGRESS REPORT:
Award Number W81XWH-14-1-0483
Grant Number: SC130298
"Recovery of breathing and forelimb function after prolonged exposure to repetitive acute intermittent hypoxia"
This report concerns research progress at the University of Florida and University in Saskatchewan, Canada.

INTRODUCTION
Our goal is to test the hypotheses that **prolonged** exposure to repetitive acute intermittent hypoxia (rAIH) in a rodent model of cervical spinal injury (C2 spinal hemisection; C2HS) elicits: recovery of breathing capacity (Aim 1) and forelimb function (Aim 2), without systemic hypertension or hippocampal pathology (Aim 3).

SPECIFIC AIMS

Aim 1 (Florida): Test the hypothesis that prolonged rAIH elicits robust and enduring improvement of breathing capacity after chronic SCI. We hypothesize that 3 to 6 months of rAIH (3 to 4 times per week; 10, 5 min episodes per exposure, 5 min intervals) elicits more robust and longer-lasting recovery of respiratory function versus daily AIH for 7 days (as published previously). We predict that functional benefits of rAIH for 3 to 6 months will persist after treatment. Experiments are performed in rats with chronic C2HS; indicators of respiratory function include baseline and maximal chemoreceptor-driven tidal volume and phrenic motor output. Rats receive intrapleural cholera toxin B fragment injections to label respiratory motor neurons and tissues harvested to assess key molecules in IH-induced respiratory motor plasticity.

Aim 2 (Saskatchewan): Test the hypothesis that prolonged rAIH combined with task specific training elicits robust and prolonged improvement of voluntary forelimb function after C2HS. Since earlier rat and human studies demonstrate that rAIH is most effective at restoring over-ground walking ability when paired with locomotor training, we will perform 3 month rAIH exposures with paired horizontal ladder walking training to elicit more robust and longer-lasting recovery of forelimb function. Experiments are performed in rats with C2HS; indicators of forelimb function include horizontal ladder walking and reach-to-grasp performance. Forelimb muscles are injected with cholera toxin B fragment to enable assessment of key molecules for IH-induced motor plasticity in relevant motor neuron pools.

Aim 3 (Florida): Test the hypothesis that prolonged rAIH (3 to 6 months) has no significant impact on hippocampal cell survival or systemic blood pressure. It is essential to know if prolonged repetitive AIH crosses a "dose" threshold, leading to the onset of pathology. We propose to assess blood pressure and markers of hippocampal apoptosis and reactive gliosis.

OVERALL PROJECT SUMMARY
Currently, the project is moving along at the projected pace, but was delayed due to: 1) the move of PI G.S. Mitchell from the University of Wisconsin to the University of Florida; 2) the need to re-establish a new laboratory in Florida, including recruiting and training personnel; and 3) the need to redo administrative approvals at the new institution, including IACUC and ACURO approval. Experiments at the University of Saskatchewan were delayed by: 1) delays in transfer of funds from Wisconsin to Florida, followed by administrative delays in creating the sub-award to the University of Saskatchewan; 2) the need to recruit and train a new postdoc to complete the proposed studies; and 3) loss of the initial rat group due to a pinworm infection. These hurdles have been overcome and the project is now proceeding at a rapid pace at both sites.

Before leaving Wisconsin, the PI used other funding to begin investigations related to this grant (which is underfunded and requires supplementation as indicated in the original application). These initial experiments are reported here because they are highly relevant to our current understanding. In specific, C2 hemisections were performed on two rat groups (both n = 6); two months later, repetitive acute intermittent hypoxia (rAIH) exposures began three times per week for six months. Two months after their final AIH exposure, plethysmography and neurophysiology experiments were performed to assess breathing ability, phrenic motor output and systemic blood pressure; after terminal experiments, the rats were perfused, and their brains and spinal cords harvested. These tissues collected at the University of Wisconsin were transported to the University of Florida, enabling us to make progress in our new laboratory once the grant was successfully transferred. We analyzed tissues for: 1) lesion size in the cervical spinal cord; 2) evidence of hippocampal pathology, including neuronal loss or reactive gliosis in the hippocampal CA1 subfield. Analysis of physiological experiments performed in Wisconsin suggests that breathing capacity, ipsilateral phrenic motor output and systemic blood pressure were unaffected two months post-rAIH. Thus, this treatment regimen did not appear

sufficient to elicit prolonged functional benefits. On the other hand, there was no evidence for pathology (hypertension or hippocampal pathology). We were not able to perform time-series assessments of ventilatory function via plethysmography since, mid-way through the study, the plethysmograph in Wisconsin ceased working; thus, it was not possible to make comparisons between pre and post exposure data. A major surprise in these experiments was that lesion volume is smaller in rats exposed to rAIH versus sham controls. Using immunohistochemical markers for GFAP, NeuN, fibroblast surface protein and serotonin, we observed preliminary evidence for: 1) new astrocytes, fibroblasts and (possible) neurons on the margins of the lesions, and 2) serotonergic fibers coursing through these new cells. These exciting preliminary findings were reported in an abstract, but require verification. We plan to investigate rAIH effects on the lesion site in tissues collected from experiments funded by this grant. Specific progress funded by this grant is outlined below.

Progress during the first year of this grant is listed in association with the Statement of Work tasks.

Specific Aim 1: Test the hypothesis that prolonged rAIH elicits robust and prolonged improvement of breathing capacity after chronic SCI.

Task 1*.* Quantify effects of rAIH on breathing in rats with chronic SCI.

*Subtask 1a. Milestone #1 Obtain Animal Use Approvals—**Completed at both sites.***

Subtask 1b. Breathing assessment of naïve rats prior to performing C2HS. Perform surgeries and then measure breathing capacity 8 weeks post surgery. (10 rats per treatment group x 4 treatment groups). **Completed.**

Subtask 1c. rAIH treatment for 3 months and then assess breathing capacity. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Completed.**

Subtask 1d. Breathing assessments post rAIH. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Completed May 3, 2017; analysis pending.**

Subtask 1e. Assess phrenic motor output and arterial blood pressure in acute neurophysiology experiments. Perfuse rats. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Completed May 3, 2017; analysis pending.**

Subtask 1f. Determine if rAIH increases expression of key proteins in spinal motor neurons. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Tissue harvest completed May 3, 2017; immunohistochemistry and quantification pending.**

Subtask 1g. Quantify hippocampal pathology. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Tissue harvest completed May 3, 2017; immunohistochemistry and quantification pending.**

Milestone #2 –Understand efficacy of rAIH for recovery of breathing function. Understand changes in key molecules within spinal respiratory and non-respiratory motor neurons. **Pending completion of tissue and data analysis in year 2.**

Specific Aim 2: Test the hypothesis that prolonged rAIH elicits robust and prolonged improvement of voluntary limb function after chronic SCI.

Subtask 2a. Conditioning and limb function assessment in the first cluster of naïve rats prior to spinal injuries (N=30 rats; 5 rats/task x 2 treatment x 3 tasks). **An initial rat group was lost due to pinworm infection. After clearing rat colony, a second rat group received training and C2HS (April, 2017).**

Subtask 2b. Measure limb function 1 month after surgery, during rAIH treatment for 3 months and after final AIH treatment in the first rat cluster. **Pending.**

Subtask 2c. Conditioning and limb function assessment in the second cluster of naïve rats prior to spinal injuries (N=30 rats; 5 rats/task x 2 treatment x 3 tasks). **Pending.**

Subtask 2d. Measure limb function 1 months after surgery, during rAIH treatment for 3 months and after

final AIH treatment in a second rat cluster. **Pending.**

Subtask 2e. Perform spinal injuries and rAIH treatment for spinal protein analysis (N=10 rats; 5 rats/task x 2 groups). **Pending.**

Subtask 2f. Quantify the expression of key proteins at regular time points during and after rAIH for 3 months in AIH and SHAM animals. **Pending.**

*Subtask 2g***.** Perform spinal injuries and AIH treatment for protein analysis in the second cluster of naïve rats (N=10 rats; 5 rats/task x 2 groups). **Pending.**

Subtask 2h. Quantify the expression of key proteins at regular time points during and after rAIH for 3 months in second cluster of rAIH and SHAM animals. **Pending.**

Milestone #3 –Understand the efficacy of 3xwAIH for recovery of forelimb recovery after chronic SCI. Understand the impact of 3xwAIH on protein expression in identified forelimb motor neurons. **Pending.**

Specific Aim 3: Test hypothesis that prolonged rAIH has no impact on blood pressure or hippocampal cells.

Task 3a. Quantify the effect of rAIH on blood pressure. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Experiments completed May 3, 2017; analysis pending.**

Subtask 3b: Quantify rAIH effects on hippocampal cells. (Note-the same rat groups are in all sub-tasks within Aims 1 & 3; 10 rats per treatment group x 4 treatment groups). **Tissue harvest completed May 3, 2017; analysis pending.**

Milestone #4 –Understand impact of prolonged rAIH on hippocampal pathology and systemic blood pressure. **Pending.**

Task 4. Analyze data and draft manuscripts concerning effects of rAIH on breathing capacity and forelimb function following chronic C2HS. **Pending (completion of analysis in Aims 1 & 3, and experiments plus analysis in Aim 2).**

KEY RESEARCH ACCOMPLISHMENTS TO DATE

We secured all necessary approvals, transferred the award to the University of Florida and the sub-award to the University of Saskatchewan, and recruited and trained personnel necessary to began experiments at both sites. The experiments are well underway. However, experiments proposed in this grant require long waiting times before data will be forthcoming, including: 1) preparing rats with chronic spinal injuries, including pre-surgical measurements, surgery, and post-surgical care, followed by the time necessary for chronic injuries (8 weeks); and 2) performing either rAIH or sham normoxia exposures. After extensive consideration, we shortened the duration of rAIH exposures to 3 months, and studied rats one day after the final AIH exposure due to difficulties in completing all proposed experiments within the allotted time. This decision was also based on preliminary experiments performed at the University of Wisconsin using other funding sources prior to the availability of funds from this grant at the University of Florida. In these preliminary experiments, rats studied two months post-rAIH (3x per week, 6 months) provided no evidence for persistent enhancement of breathing capacity (all groups had recovered full tidal volume generating ability) or ipsilateral phrenic motor output. There was also no evidence for systemic hypertension or hippocampal (CA1) pathology. However, we did see striking (and surprising) preliminary evidence that prolonged rAIH: 1) reduced volume of the injury site by inducing cell proliferation (astrocytes, fibroblasts and possible neurons); and 2) enabled serotonergic axon passage across these new cellular “bridges.” These exciting and unanticipated preliminary findings (with inadequate n values) will be verified in tissues collected with funding from this grant.

To date, all proposed animal work, including surgeries, rAIH or sham exposures, plethysmography and neurophysiology have been completed at the Florida site (Aims 1 & 3). Extensive data analyses will continue during the second year of this grant, including analysis of breathing capacity (plethysmography), terminal neurophysiology, phrenic motor neuron neurochemical changes, and characterization at the injury site. After initial delays, experiments at the Saskatchewan site are underway, and completion of animal work is anticipated during this fiscal year.

PROBLEMS

Dr. Mitchell's departure from the University of Wisconsin to the University of Florida put us behind schedule last year, making a future request for a no cost extension likely. At the Saskatchewan site, there were two major delays: 1) transfer of funds from UW to UF, and then from UF to the University of Saskatchewan; and 2) the first group of rats was terminated due to pinworm infection.

CONCLUSIONS

We made good progress in accordance with our experimental plan, although we experienced delays related to Dr. Mitchell's move to University of Florida. We made a major effort to catch up and have completed all rat procedures at the Florida site. We anticipate greater understanding concerning the efficacy and safety of rAIH as a potential therapeutic modality after completion of our physiological and tissue analyses.

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:

Santiago-Moreno, J.G., I. Satriotomo, B.J. Dougherty, S. Springborn, E. Kopp, L. Sullivan and G.S. Mitchell (2017). Repetitive acute intermittent hypoxia affects lesion volume after cervical spinal injury. *FASEB Journal* (abstract, in press).

INVENTIONS, PATENTS AND LICENSES: None

REPORTABLE OUTCOMES: None, pending completion of our studies.

OTHER ACHIEVEMENTS: None

APPENDICES: None

Recovery of breathing and forelimb function after prolonged exposure to repetitive acute intermittent hypoxia SC130298

PI: Gordon Mitchell, PhD **Org:** University of Florida

Award Amount: $450,000

Study/Product Aim(s)

• The goal is to test the hypothesis that repetitive exposure to acute intermittent hypoxia (rAIH) elicits persistent respiratory and non-respiratory motor recovery without pathology in rats with chronic cervical spinal hemisections. We hypothesize that rAIH:

- **Hypothesis 1**: improves breathing capacity;
- **Hypothesis 2**: improves horizontal ladder walking performance;
- **Hypothesis 3**: does not elicit hippocampal pathology or systemic hypertension.

Approach

• Breathing capacity and ladder walking ability will be compared with and without rAIH delivered over months.

• At the end of exposures, measurements will be made of breathing capacity, phrenic motor output and systemic blood pressure.

•Rats will be perfused and brain/spinal cord tissues examined for: 1) injury site; 2) protein expression in phrenic motor neurons; and 3) evidence of hippocampal gliosis or cell death.

Accomplishments:

- Regulatory requirements fulfilled and laboratory staff trained.
- In Florida, 60 were exposed to rAIH or sham normoxia with measurements of breathing capacity and terminal neurophysiology. Analysis of tissues is underway.
- In Saskatchewan, a new postdoc completed the first cohort of the ladder walking study after an unexpected setback.
- Preliminary data suggest an unanticipated effect—that rAIH reduces injury volume size, filling the zone with new neurons, glia and fibroblasts, and that serotonergic axons pass through this new cellular "bridge." Serotonin terminal density in the phrenic motor nucleus remains elevated. These preliminary findings require verification.

Timeline and Cost

Activities	16	17-18
After 4 rat groups receive cervical hemisections and/or sham surgery, they are exposed to rAIH beginning two months post-injury. Throughout, assessments of breathing capacity or ladder walking ability will be made.		
At the end of exposures, phrenic motor output will be assessed, the rats sacrificed and perfused tissues will be removed.		
Analysis of brain and spinal cord tissues. Data analysis. Preparation of manuscripts		
Estimated budget ($K)	$225,000	$225,000

Updated: (5/08/2017)

Goals/Milestones

CY16 Goal – complete administrative requirements

☑ ACURO

☑ train personnel

CY16 Goals – initiate rat treatments to cervical hemisection and raIH.

☑ Perform Cervical hemisections

☑ exposed subset of rats to IH

CY17-18 Goals – Data collection/manuscript preparation

☐ Perform (done) and analyze ventilatory measurements

☐ Perform and analyze changes in ladder walking ability

☐ Perform (done) and analyze acute neurophysiological experiments

☐ Analyze brain and spinal cord tissues via neurochemical/immunofluoresence.

☐ Prepare manuscripts

Comments/Challenges/Issues/Concerns

- After delays due to the grant transfer, ACURO approval and to train new staff, we have made good progress. We may need to extend into a third year (NCE).

Budget Expenditure to Date

Projected Expenditure: $450,000 (direct; as per original budget)

Actual Expenditure: ~$400,000 direct costs (obligated)